Dexamethasone induces caveolin-1 in vascular endothelial cells: implications for attenuated responses to VEGF

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Igarashi J, Hashimoto T, Shoji K, Yoneda K, Tsukamoto I, Morie T, Kubota Y, Kosaka H. Dexamethasone induces caveolin-1 in vascular endothelial cells: implications for attenuated responses to VEGF. Am J Physiol Cell Physiol 304: C790–C800, 2013.—Steroids exert direct actions on cardiovascular cells, although underlying molecular mechanisms remain incompletely understood. We examined if steroids modulate abundance of caveolin-1, a regulatory protein of cell-surface receptor pathways that regulates the magnitudes of endothelial response to vascular endothelial growth factor (VEGF). Dexamethasone, a synthetic glucocorticoid, induces caveolin-1 at both levels of protein and mRNA in a time- and dose-dependent manner in pharmacologically relevant concentrations in cultured bovine aortic endothelial cells. Aldosterone, a mineralocorticoid, but not the sex steroids 17β-estradiol, testosterone, or progesterone, elicits similar caveolin-1 induction. Caveolin-1 induction by dexamethasone and that by aldosterone were abrogated by RU-486, an inhibitor of glucocorticoid receptor, and by spironolactone, a mineralocorticoid receptor inhibitor, respectively. Dexamethasone attenuates VEGF-induced responses at the levels of protein kinases Akt and ERK1/2, small-G protein Rac1, nitric oxide production, and migration. When induction of caveolin-1 by dexamethasone is attenuated either by genetically by transient transfection with small interfering RNA or pharmacologically by RU-486, kinase responses to VEGF are rescued. Dexamethasone also increases expression of caveolin-1 protein in cultured human umbilical vein endothelial cells, associated with attenuated tube formation responses of these cells when cocultured with normal fibroblasts. Immunohistochemical analyses revealed that intraperitoneal injection of dexamethasone induces endothelial caveolin-1 protein in thoracic aorta and in lung artery in healthy male rats. Thus steroids functionally attenuate endothelial responses to VEGF via caveolin-1 induction at the levels of signal transduction, migration, and tube formation, identifying a novel point of cross talk between nuclear and cell-surface receptor signaling pathways.

steroid hormones that act through nuclear glucocorticoid, mineralocorticoid, and sex steroid receptors play essential roles in the maintenance of animal body homeostasis. In pathophysiological contexts, agonists for these steroid receptors are capable of exerting direct actions on cardiovascular cells, besides modulating water balance and blood pressure. For example, glucocorticoid receptor (GR) agonists downregulate endothelial nitric oxide synthase (eNOS) expression in rodent blood vessels (38) and in cultured endothelial cells (39). Clinically, hypercortisolemia in Cushing’s syndrome is correlated with higher overall cardiovascular risk (26, 35) and with attenuated endothelium-dependent flow-mediated vasodilation responses in forearm arteries (1, 4). Mineralocorticoid receptor (MR) agonists also exert direct cardiovascular actions, for example, by decreasing glucose-6-phosphate dehydrogenase activity of endothelial cells (20) or by inducing eNOS “uncoupling” (28). It has clinically been known that patients suffering from primary aldosteronism exhibit a cardiovascular complication rate out of proportion to blood pressure levels seen in those suffer from essential hypertension (6). In contrast, a sex steroid 17β-estradiol increases eNOS at the levels of enzyme activity and mRNA and protein expression levels, leading to favorable outcomes on the vasculature (17). Cardiovascular actions of steroid receptor agonists may also be related to the modulation of angiogenesis, formation of new blood vessels from the preexisting ones. For example, inhaled corticosteroid effectively attenuates pathological angiogenesis in chronic airway diseases (40). Addition of corticosteroid to infantile hemangioma-derived stem cells suppresses vascular endothelial growth factor (VEGF) production, thereby leading to attenuated tumor growth in vivo (13). VEGF represents a well-characterized polypeptide growth factor acting through specific receptor tyrosine kinases expressed on the endothelial cell surface, leading to numerous responses. However, how steroids may regulate functions of vascular endothelial cells, including those modulated by VEGF, has remained incompletely understood.

In vascular endothelium, plasmalemmal caveolae, small and flask-shaped invaginations, serve as key signal transducing microdomains (30). These microdomains are enriched in various endothelial signaling proteins, including receptors such as VEGF receptor 2 (VEGFR2), protein kinases such as Akt as well as MAP kinases ERK1/2, and effector molecules such as eNOS. Caveolins are the constituent proteins of caveolae that interact with and modulate functions of caveolae-targeted proteins (30). It appeared interesting to us that some of the steroids influence the abundance of caveolin-1 isoform. For example, progesterone and testosterone increase caveolin-1 expression in breast (31) and prostate (22) cancer cells, respectively. Caveolin-1 is upregulated by the GR agonist dexamethasone in rat-derived lung epithelial cell lines (2). We therefore examined hypotheses that steroids modulate expression levels of caveolin-1 and that induction of caveolin-1 by steroids is associated with perturbed endothelial responses to VEGF.
MATERIALS AND METHODS

Reagents. Antibodies and related compounds were commercially obtained as follows: anti-caveolin-1, anti-eNOS, and anti-ERK1/2 monoclonal antibodies were from BD Biosciences (San Jose, CA); anti-phospho VEGFR2 antibodies (Tyr1175), anti-phospho-Akt antibody (Ser473), anti-Akt polyclonal antibody, anti-phospho-eNOS antibody (Ser1179), and anti-phospho-ERK1/2 antibody (Thr202/Tyr204) were from Cell Signaling Technologies (Beverly, MA). Polyclonal antibodies directed to actin and to von Willebrand factor (vWF) were from Santa Cruz (Santa Cruz, CA). SuperBlock reagents, SuperSignal substrates for chemiluminescence detection, Restore Western Blot Striping Buffer, and secondary antibodies conjugated with horseradish peroxidase were from Pierce (Rockford, IL). FITC-conjugated antibody to rabbit IgG, Cy3-conjugated streptavidin, and biotin-conjugated anti-mouse IgG were from DAKO (Glostrup, Denmark). TO-PRO 3 was from Invitrogen.

Bovine aortic endothelial cells (BAECs) were obtained from Cell Systems (Kirkland, WA). DME, LipofectAMINE 2000, and OptiMEM were from Invitrogen (Carlsbad, CA). FBS was purchased from Hyclone (Logan, CT). Human umbilical vein endothelial cells (HUVEC), HuMedia-EG2 culture medium, and tubeule staining kit for CD31 were obtained from Kurabo (Osaka, Japan). Protease Inhibitor Cocktail III was from Merck (Whitehouse Station, NJ). An angiogenesis kit that contained a coculture system of HUVEC and normal human fibroblasts, supplemented with culture medium and accessory reagents, was also from Kurabo. RNeasy mini columns were from Qiagen (Valencia, CA). Taq DNA polymerase was from Promega (Madison, WI). The Rac1 activity assay kit was from Cytoskeleton (South Acoma, CO). An automated HPLC system ENO-20 was from Eicom (Kyoto, Japan). The CytoSelect 24-well cell migration assay kit (8 μm, Colorimetric Format) was from Cell Biolabs (San Diego, CA). Male Wister rats (8-wk-old) were obtained from Japan SLC (Shizuoka, Japan). A tissue-freezing medium, Tissue Tek OTC compound, was from Sakura Finetechnical (Tokyo, Japan). Protein concentration was determined by a protein assay bicinchoninate kit from Nacalai Tesque (Kyoto, Japan). All materials were from Sigma (St. Louis, MO) unless otherwise stated.

Cell culture and drug treatment. BAEC and HUVEC were maintained in culture as described previously (14, 36). HUVEC were used between passages 3 and 6. Steroids and steroid receptor antagonists were resolved into ethanol and were stored at −80°C. After reaching subconfluence, both cells were cultured in medium containing 1% of FBS for 2 days together with various drugs before being used for experiments. The final concentration of solvents did not exceed 0.1%. DNA preparation and amplification by real-time quantitative RT-PCR. Total RNA was isolated from BAEC using the RNeasy mini column (Qiagen) (15). One microgram of total RNA was transcribed into cDNA using random hexamer and reverse transcriptase in a total volume of 20 μl. The reverse transcription was performed at 37°C for 90 min and then at 70°C for 15 min. Quantitative RT-PCR was performed by using SYBR Premix Ex Taq II system (Takara Biotechnology Shiga, Japan) and melting curve analysis using StepOne Plus Real-time PCR system (Applied Biosystems) with 18S rRNA as a reference gene. We used the Perfect real-time supporting system (Takara) to design primers specific to bovine caveolin-1 (primer set ID: BA050199) and 18S rRNA (primer set ID: BA030502). After an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 94°C for 10 s and annealing and extension at 60°C for 40 s for 50 cycles. The quantities of amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green II (Takara) and the ROX Reference Dye (Takara). The copy number in each PCR product was defined based on a standard curve. A calibration curve was constructed by plotting the PCR threshold cycle number at which the fluorescent signal generated during the replication process passes above a threshold value against known amounts of cDNA. Caveolin-1 mRNA expression levels were normalized with 18S rRNA mRNA level.

Immunoblot analyses. Immunoblot analyses were performed as described previously (15). After being washed with ice-cold PBS, cells were harvested and lysed into cell lysis buffer containing 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 150 mM NaCl, 1 mM NaVO4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and a mixture of protease inhibitors. Proteins were denatured, size-fractionated on sodium dodecyl sulfate polyacrylamide gels, and transferred to nitrocellulose membranes. The resulting membranes were blocked and incubated with various primary antibodies, followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies, performed in TBS (pH 7.4) supplemented with 0.1% Tween-20. Immunoreactive signals were visualized using Pierce SuperSignal substrates for chemiluminescence detection with exposure to standard X-ray films (Fuji, Tokyo, Japan). In some experiments, antibodies used in the immunoblot analyses were removed from membranes using Restore Western Blot Striping Buffer and reblotted with different antibodies.

Isolation of caveoleae-enriched fractions. Caveoleae-enriched fractions were separated by using ultracentrifugation with a discontinuous sucrose gradient system essentially as previously described (15, 33). Briefly, BAEC from two 100-mm dishes were scraped together into 2 ml of “carbonate buffer” containing 500 mM sodium carbonate (pH 11), 25 mM 2-(N-morpholino)ethanesulfonic acid, and 150 mM NaCl, and the cells were homogenized and sonicated. After an aliquot of whole cell lysate had been saved, the resulting cell suspension was brought to 45% sucrose (w/v) by addition of 2 ml of carbonate buffer containing 90% sucrose and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the 45% sucrose bed by addition of 4 ml each of 35 and 5% sucrose solutions prepared in carbonate buffer. After centrifugation using a CPS40T rotor (Hitachi, Tokyo, Japan), 12 × 1 ml fractions were collected starting at the top of each gradient. An equal volume of each fraction was analyzed by SDS-PAGE and immunoblotting.

Transfection with small interfering RNA. BAEC were transiently transfected with 1 nM of small interfering RNA (siRNA; Ref. 15). Sequences of control and caveolin-1-specific siRNA were exactly as described previously (12, 15). Five hours after transfection, cells were recovered in a growth medium overnight. Then, they were incubated for 48 h in medium containing 1% FBS, together with either dexamethasone or vehicle.

Rac1 activity assay. Rac1 activity in BAEC was assessed as described previously (15) using a commercially available pull-down assay kit, in which GTP-bound (activated) form of Rac1 was precipitated using beads conjugated with the glutathione S-transferase-tagged p21-binding domain of p21 activated kinase I.

Measurement of nitrite levels in culture media. Release of nitric oxide from BAEC for 6 min after addition of VEGF was examined by measurement of nitrite accumulation in the phenol red-free culture media as described (14). The degrees of nitrite accumulation were expressed as picomoles per milligrams of protein per minute.

Cell migration assay. The migration assay used a commercially available assay kit (15). Briefly, BAEC were tripinnized and seeded onto a 24-well insert membrane medium containing 0.4% FBS. Cells that had migrated across the membranes toward chemotacticants were stained with a blue-color dye. They were then dissolved into a lysis solution and subjected to quantification by measuring the optical density at 560 nm.

Tube formation assay. The tube formation activity of cultured endothelial cells was assessed using a commercially available coculture system of HUVEC with normal human fibroblasts as described previously (36). Ten days following incubation periods with dexamethasone (10 μM) or vehicle, endothelial cells were stained and identified with an anti-CD-31 antibody. The area of the formed tube was measured by the ImageJ program. Four pictures from each well
were provided for the estimation. Culture media and reagents were exchanged every 3 days during the assay.

**Animal studies and immunofluorescence microscopy.** Immunostaining was performed as described previously (41). Twelve of the 8-wk-old male Wistar rats were housed one rat per cage with temperature and light control (25°C and 12:12-h light-dark cycle). Six each of them were intraperitoneally injected with dexamethasone (0.1 mg·kg body wt⁻¹·day⁻¹) or equivalent volume of ethanol in every morning, respectively. They were killed exactly 48 h after the initial dexamethasone administration using pentobarbital (50 mg/kg body wt ip injection). All rat lung and descending aorta tissues were embedded in a tissue-freezing medium and frozen in liquid nitrogen. Cryostat sections were incubated with primary antibodies overnight at 4°C, and the immunoreactive signal was detected with FITC-conjugated antibody to rabbit IgG or a combination of antibodies against bixin conjugated anti-mouse IgG. TO-PRO-3 was used for nuclear detection. Immunofluorescent images were viewed with LSM 700 confocal laser microscope (Carl Zeiss, Jena, Germany). Immunoreactive signals corresponding to endothelial caveolin-1 in a given slice derived from either thoracic aorta or from lung artery were captured using a fluorescence microscope BioRevo BZ9000 (Keyence, Tokyo, Japan) and were quantified using the Keyence software, Dynamic Cell Count. These experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Kagawa University.

**Other methods.** All cellular experiments were performed at least three times. Mean values for individual experiments are expressed as means ± SE. Statistical differences were analyzed by ANOVA followed by Scheffé’s F test or by Student’s t-test where appropriate using Statcel3 (OMS, Saitama, Japan). A P value <0.05 was considered statistically significant.

**RESULTS**

We started our exploration by treating BAEC, an archetypal model of endothelial cell culture, with dexamethasone, a synthetic GR agonist. Quantitative RT-PCR assays revealed that dexamethasone (for 48 h) led to dose (Fig. 1A)- and time (Fig. 1B)-dependent increases in caveolin-1 mRNA abundance. We also subjected the same cDNA templates to conventional semiquantitative RT-PCR assays and obtained similar results that caveolin-1 mRNA abundance was augmented with dexamethasone treatment (data not shown). Additionally, we noted that dexamethasone did not alter the amounts of BAEC transcripts that encode GAPDH. We next studied the effects of dexamethasone on caveolin-1 protein amounts. Dexamethasone induced a dose-dependent upregulation of caveolin-1 at a level of protein expression (Fig. 2). We tested the effects of several other steroid receptor agonists and found that aldosterone, a MR agonist, but not the sex steroids 17β-estradiol, testosterone, or progesterone, elicited induction of caveolin-1 protein albeit to a lesser extent than does dexamethasone (Fig. 2). Not only dexamethasone but also cortisol and corticosterone, which represent endogenously produced glucocorticoids in various animal species (10), similarly upregulated caveolin-1 abundance in BAEC (data not shown).

Because caveolin-1 protein undergoes a variety of posttranslational modifications including oligomerization and translocates to caveolea microstructures of peripheral plasma membrane (reviewed in Ref. 7), it was of interest to explore whether or not caveolin-1 is properly targeted to caveola-rich fractions even after being upregulated by dexamethasone. For this sake, we performed a well-established detergent-free subcellular fractionation protocol with a discontinuous sucrose density system (33) to analyze BAEC lysates (15) with or without dexamethasone. The results indicate that caveolin-1 signals were detected only at the interface between 5 and 35% sucrose solutions, which represent caveola-rich fractions.
(Fig. 3A). Thus caveolin-1 seems to be targeted to caveolae-like fractions even in the presence of dexamethasone after undergoing similar post-transcriptional modifications with vehicle-treated cells. We then performed a time-course assay in which BAEC were treated with 1 μM of dexamethasone for various time points, followed by immunoblot analyses. Results indicate that dexamethasone led to time-dependent increases in caveolin-1 protein abundance (Fig. 3B). Expression levels of several other endothelial proteins, including actin, VEGFR2, eNOS, protein kinases ERK1/2 as well as Akt, a small G-protein Rac1, did not change over these dexamethasone treatment protocols (see Figs. 3B, 4, and 5). We tested whether or not RU-486, a GR antagonist, and spironolactone, a MR inhibitor, counteracts steroids-induced upregulation of caveolin-1 protein in BAEC. Dexamethasone and aldosterone at 1 μM increased the abundance of caveolin-1 to the similar degrees as we observed in Fig. 2; however, promotion of caveolin-1 expression was not seen in the presence of RU-486 and spironolactone, respectively (Fig. 4, A and B). Collectively, these results serve to demonstrate that agonists for GR and MR, but not those for sex steroid receptors, are capable of upregulating the expression of caveolin-1 at the levels of protein and mRNA in BAEC.

Caveolin-1 is a key scaffolding protein of caveolae microdomains that modulates amplitudes of endothelial cell responses to various extracellular stimuli, including polypeptide growth factors such as VEGF (19). We therefore explored functional consequences of steroid induction of caveolin-1. We examined various responses of BAEC to VEGF that had been treated with or without dexamethasone for 48 h. We first examined the protein phosphorylation responses. As shown in Fig. 5A, the degrees of VEGF-promoted protein phosphorylation in VEGFR2 (Tyr1175), kinases Akt (Ser473), ERK1/2 (Thr202/Tyr204), and eNOS (Ser1179) were markedly attenuated in cells pretreated with dexamethasone that expressed higher amounts of caveolin-1 protein. When dexamethasone-elicited induction of caveolin-1 protein had been pharmacologically counteracted by RU-486, attenuation of these phosphorylation responses to VEGF was reversed (Fig. 5B). We also took genetic knockdown approach using previously established siRNA specific to bovine caveolin-1 (12) in combination with dexamethasone. When upregulation of caveolin-1 protein by dexamethasone was abrogated by siRNA transfection, attenuation of phosphorylation responses in VEGFR2, Akt ERK1/2, and eNOS proteins were completely recovered (Fig. 5C), although neither treatment with dexamethasone nor transfection with caveolin-1 siRNA affected abundance of total VEGFR2, Akt ERK1/2, or eNOS. Thus ability of dexamethasone to attenuate VEGF-elicited protein phosphorylation responses derives from upregulation of caveolin-1. Rac1 is a small G-protein that plays a pivotal role in mediating VEGF signaling pathways (11). Figure 6A indicates that Rac1 activation by VEGF was reduced in dexamethasone-pretreated cells compared with vehicle. eNOS is a downstream effector that is activated by VEGF under the control of upstream protein kinases and small G proteins (21). Figure 6B shows that stimulation with VEGF led to higher levels of nitric oxide production and that pretreatment with dexamethasone markedly abrogated it. When induction of caveolin-1 by dexamethasone was inhibited by siRNA transfection, attenuation of VEGF-elicited nitric oxide production was markedly counteracted (Fig. 6C). These data indicate that dexamethasone, which elevates caveolin-1 abundance, attenuates VEGF-activated signal transduction pathways at multiple levels including protein phosphorylation, Rac1 activation, as well as eNOS activation.

Migration and tube formation of endothelial cells in response to VEGF represent important physiological steps.
Fig. 3. Characterization of dexamethasone-induced upregulation of caveolin-1 protein abundance in BAEC. A: results of subcellular fractionation assay of BAEC treated with or without Dex (at 1 μM for 48 h). Sonicated cell lysates from BAEC were separated using a discontinuous sucrose gradient system as described in MATERIALS AND METHODS. An equal volume from each fraction was separated by SDS-PAGE, followed by immunoblot analysis using an antibody directed against caveolin, as indicated at top. An aliquot of whole cell lysate had been withdrawn before subcellular fractionation and was separately subjected to immunoblots as shown at bottom. Shown are representative of 3 independent experiments that yielded equivalent data. Note that Cav-1 protein in both vehicle- and Dex-treated BAEC was specifically recovered at the interface between the 5% and 35% sucrose gradient, representing the caveolae-enriched fractions. B: results of time course assay in which BAEC were treated with 1 μM of Dex for the indicated durations. Cellular proteins were probed with antibodies directed to Cav-1, endothelial nitric oxide synthase (eNOS), actin, and Akt. Top: data are representative of 4 independent experiments that yielded equivalent results. Bottom: results of densitometric analyses from pooled data, plotting the fold increase of the degree of expression levels of Cav-1 and eNOS at the indicated time point, relative to the signals obtained in the absence of dexamethasone. *P < 0.05 vs. cells treated with vehicle alone.

during angiogenic processes evoked by the growth factor (29). Using a modified Boyden chamber assay, we found that BAEC preexposed to dexamethasone exhibited lower magnitudes of migration both toward VEGF and toward serum (Fig. 7, A and B). We sought to explore the effects of dexamethasone in a human-derived endothelial cell culture model, HUVEC. Dexamethasone led to time-dependent augmentation of caveolin-1 protein abundance in HUVEC monolayer (Fig. 7C). Unlike in BAEC, dexamethasone attenuated eNOS protein expression. After confirming that dexamethasone induces caveolin-1 in HUVEC, we explored the effects of dexamethasone in tube forming activity using a commercially available and previously established coculture system of HUVEC with normal human fibroblasts (36). In this system, cells were incubated for 10 days in the presence and absence of dexamethasone. The degrees of endothelial tube formation were determined by means of immunostaining using an anti-CD31 antibody. Figure 7D indicates that the magnitude of tube formation by HUVEC in the presence of dexamethasone markedly decreased to approximately half of that seen with the vehicle. Together, these results demonstrate that endothelial cells pretreated with dexamethasone that express higher amounts of caveolin-1 exhibit attenuated responses to VEGF not only at the level of signal transduction but also at more distal levels of motility, i.e., migration and tube formation.

To explore whether or not steroids are able to upregulate caveolin-1 protein expression in vascular endothelium of living animals, we isolated thoracic aorta and lung tissues from normal male rats treated with or without dexamethasone and double-stained them with anti-caveolin-1 and anti-vWF antibodies. Caveolin-1 distributed in aorta and lung arterial cells (Fig. 8). When dexamethasone was applied for 2 days, enhanced expression of caveolin-1 was observed. Quantitative microscopic analyses revealed that endothelial caveolin-1 immunoreactive signals significantly increased by 63 ± 29% in thoracic aorta (P < 0.05 vs. vehicle) and tended to increase by 62 ± 13% in lung artery (P < 0.1). In contrast, expression of vWF of endothelial cells did not change at before and after dexamethasone treatment. These data indicate that the expression of caveolin-1 is upregulated at both aorta and lung arterial endothelial cells after dexamethasone treatment in living animals as well as in cultured endothelial cells. When whole tissue extracts were analyzed for caveolin-1 mRNA (lung and aorta, conventional RT-PCR) and protein (lung, immunoblot), we did not observe differences between dexamethasone- vs. vehicle-treated animals (data no shown). These results suggest that caveolin-1 induction by dexamethasone under these stimulation protocols occurred specifically in vascular endothelium, rather than taking place in the bulk of cell types.
Several nonendothelial cell types, for example, in breast (31) as transcription, or degradation pathways (reviewed in Ref. 7). Some other than transcription for example by translation, posttranslatability that steroids modulate caveolin-1 expression at the levels currently known in endothelial cells. There remains the possibility that steroids modulate caveolin-1 expression at the levels other than transcription for example by translation, posttranscription, or degradation pathways (reviewed in Ref. 7). Some sex steroids are capable of modulating caveolin-1 expression in several nonendothelial cell types, for example, in breast (31) as well as in prostate (22) cancer cells. Thus precise molecular mechanisms whereby steroids regulate caveolin-1 expression, which are beyond the scope of current studies, remain to be elucidated both in endothelial- and nonendothelial cells.

Dexamethasone upregulates caveolin-1 in rat-derived lung epithelial cell lines (2) and in pancreatic acinar cells (24). Adipocytes express dramatically higher levels of caveolin-1 when differentiated by a hormonal mixture including dexamethasone (32). Thus these earlier observations indicate that glucocorticoid-induction of caveolin-1 is not limited to vascular endothelial cells. However, our data indicate that in whole rat organ homogenates expression levels of caveolin-1 mRNA (lung and aorta) and protein (lung) do not change 48 h after dexamethasone (data not shown), suggesting that dexamethasone does not largely affect caveolin-1 abundance in nonendothelial cell types under current protocols. Notably, functional consequences of caveolin-1 upregulation by steroids have remained completely unexplored in any cell types. We therefore sought to determine if dexamethasone leads to perturbed responses of vascular endothelial cells to extracellular stimuli.

We focused on VEGF because this angiogenic growth factor represents a pivotal regulator of endothelial functions (29) and because VEGF receptor signaling is modulated by caveolae (19, 23). VEGF activates a wide array of endothelial signal transduction pathways, starting from activation/phosphorylation of cognate receptor tyrosine kinases (VEGFR2), activation/phosphorylation of downstream protein kinases including Akt and ERK1/2, activation of a key small G-protein Rac-1, as well as eNOS-dependent nitric oxide production associated with Akt-mediated phosphorylation of caveolin-1 (37), the functions of these genomic elements are not currently known in endothelial cells. There remains the possibility that steroids modulate caveolin-1 expression at the levels other than transcription for example by translation, posttranscription, or degradation pathways (reviewed in Ref. 7). Some sex steroids are capable of modulating caveolin-1 expression in several nonendothelial cell types, for example, in breast (31) as well as in prostate (22) cancer cells. Thus precise molecular mechanisms whereby steroids regulate caveolin-1 expression, which are beyond the scope of current studies, remain to be elucidated both in endothelial- and nonendothelial cells.

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**Fig. 4.** Effects of steroid receptor antagonists on caveolin-1 upregulation induced by dexamethasone and aldosterone. A: results of protein immunoblot analyses in which effects of RU-486, a glucocorticoid receptor (GR) inhibitor, on Dex-mediated upregulation of endothelial Cav-1 protein abundance were examined. BAEC had been treated with RU-486 (1 μM for 30 min) or vehicle; an aliquot of Dex stock solution (or vehicle) was then added to the cultures to achieve a final concentration of 1 μM, as indicated. Incubation proceeded further for 48 h and protein samples were harvested and subjected to immunoblot analyses as above. Left: representative images. Right: results of densitometric analyses from pooled data, plotting the fold increase of the degree of expression levels of Cav-1, relative to the signals obtained in the absence of Dex and RU-486. B: spironolactone (Spi), a mineralocorticoid receptor inhibitor, and Aldo (both at 1 μM) were employed instead of RU-486 and Dex; n = 4 in A and B.
experiments we focused on one micromolar of dexamethasone, as one of the signaling events is alike attenuated. The eNOS protein. Our results show that when endothelial cells were treated with Dex followed by VEGF, BAEC had been incubated with Dex (1 μM for 48 h) or vehicle and then they were treated with VEGF (5 ng/ml). Following treatment with VEGF for the times indicated, cellular proteins were subjected to immunoblot assays, probed with antibodies directed to phospho-VEGFR2 (Tyr1175), phospho-Akt (Ser473), phospho-eNOS (Ser1179), phospho-ERK1/2 (Tyr202/Thr204), and Cav-1. Equal loading of samples was confirmed by reprobing the immunoblots with antibodies against (total) VEGF and Dex. Open and closed circles represent values obtained with and without pretreatment with Dex, respectively. *P < 0.05 vs. VEGF (--), †P < 0.05 vs. Dex (--); n = 4. B: cells had been pretreated with RU-486 and/or Dex (as in Fig 4A), followed by VEGF (5 ng/ml for 10 min). After the addition of VEGF, cells were harvested and subjected to immunoblot analyses and densitometry as above, normalizing the values to those obtained in the absence of RU486, Dex and VEGF. *P < 0.05 vs. VEGF (--), †P < 0.05 vs. RU486 (--), ‡P < 0.05 vs. Dex (--); n = 4. C: BAEC were transfected with either control siRNA [indicated as Cav-1 siRNA (--)] or that directed to caveolin-1 [Cav-1 siRNA (+)] before dexamethasone (1 μM for 48 h) followed by VEGF (5 ng/ml for 5 min). Cells were then subjected to immunoblot analyses and densitometry as above, normalizing the values to those obtained in the absence of Dex and VEGF in control siRNA-treated cells. *P < 0.05 vs. VEGF (--), †P < 0.05 vs. control siRNA. ‡P < 0.05 vs. Dex (--).
1-specific siRNA, counteracts attenuation of protein phosphorylation responses to VEGF induced by dexamethasone, concomitantly with decreases in caveolin-1. Caveolin-1 protein is supposed to be specifically targeted to plasmalemmal caveolae after completing its oligomerization processes (7). Our subcellular fractionation assays revealed that the caveolin-1 protein in BAEC is specifically targeted to caveole-enriched fractions regardless of dexamethasone treatment. Thus the steroid agent appears to alter VEGF responses primarily by augmenting the abundance of caveolin-1 protein rather than by influencing its subcellular location and/or oligomerization status. An earlier report shows that in heterologous expression systems of 293T human fibroblast cell line, caveolin-1 acts as a direct negative regulator of VEGFR2 tyrosine kinase (19). Caveolin-1 also plays inhibitory roles in VEGF signal transduction pathways at the level of eNOS enzyme (27) and at that of upstream Rac1-Akt signaling axis (11, 12, 21). Endothelial-specific overexpression of caveolin-1 leads to impaired vascular permeability and angiogenic responses to VEGF in mice (3). When taken together with these earlier observations, the present results are consistent with a hypothesis that it is the abundance of caveolin-1 protein that plays a major role to determine the amplitudes of endothelial signaling responses to VEGF, under the control of nuclear steroid receptors. In a broader perspective, however, the roles of caveolin-1 in modulating the cellular signal transduction pathways appear to be highly complex and context dependent. For example, a stable knockdown of caveolin-1 in NIH-3T3 cells leads to augmented ERK1/2 activation (9). In contrast, in cultured endothelial cells derived from caveolin-1 null mice, VEGF-induced ERK1/2 phosphorylation responses are abrogated, rather than hyperactivated (34). Although our results are in line with the former example, these earlier studies suggest that one needs a caution in extrapolating the findings obtained in a given cellular system to the other in terms of caveolin-1 actions on receptor signal transduction pathways.

We have tested the effects of dexamethasone on migration as well as tube formation, both representing key angiogenic processes of vascular endothelial cells. Modified Boyden chamber assay revealed that cells pretreated with dexamethasone that express more abundant caveolin-1 protein display significantly lower degrees of migratory responses evoked both by VEGF and by 10% FBS in BAEC, suggesting that upregulation of caveolin-1 by dexamethasone is associated with perturbed migration toward VEGF and also likely toward some other migratory response-related bioactive substances included in the FBS. By using a previously

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Fig. 6. Effects of dexamethasone on VEGF-elicited Rac1 activation and nitric oxide production in BAEC. **A**: results of Rac1 activity assay. BAEC had been incubated with Dex (1 μM for 48 h) or vehicle, then they were treated with VEGF (10 ng/ml for 1 min). Rac activity in the cell lysate was measured by means of pull-down of GTP-bound Rac1. Precipitated Rac1 was quantified in immunoblots probed with a specific monoclonal antibody. Aliquots of total cell lysates were subjected to immunoblots in separate gels using antibodies specific to Rac1 and Cav-1, as indicated; n = 3. **B** and **C**: results of nitrite measurement. **B**: BAEC were treated with VEGF (10 ng/ml for 6 min); some cells had been pretreated with Dex (1 μM for 48 h). Culture media were then collected and subjected to nitrite measurement as described in the text; n = 4. **C**: BAEC had been transfected with either control or Cav-1 specific siRNA, indicated as Cav-1 siRNA (−) or (+), respectively, before being treated with VEGF. n = 5.
established coculture model of HUVEC with normal human fibroblasts, we showed that incubation of these cells with dexamethasone for 10 days leads to attenuated endothelial tube formation activity, identified with an anti-CD31 antibody 10 days after the incubation with Dex (1 μM) or vehicle. This implies that dexamethasone does not do so in BAEC. This suggests that aortic rings from animals sensitized with antibody to caveolin-1 (18), suggesting some functional linkages of VEGF and steroid pathways in humans. We propose that regulation of caveolin-1 abundance by nuclear steroid receptor pathways may uncover a novel mechanism at which exposure to excess steroids, including agonists for GR and MR, leads to perturbed sensitivities of vascular endothelium to angiogenic growth factors such as VEGF. Although our experiments using normal male rats demonstrate that administration of a GR agonist can upregulate caveolin-1 in living animals, it remains to be seen if this were the case with human subjects.

In these experimental settings, expression levels of several other proteins including actin, VEGFR2, Akt, ERK1/2, and Rac1 did not change over various stimulation protocols with steroid receptor agonists, suggesting that steroid induction of endothelial caveola-related proteins is relatively specific to caveolin-1. In some immunoblot assays we exploited Akt as a loading control because in these panels we performed phospho-Western analyses of this protein, because steroids did not alter its expression, and because the anti-Akt antibody used in this study yielded relatively strong and specific immunoreactive signals in our system. As to eNOS, dexamethasone leads to significant down-regulation of its protein expression level in HUVEC, although it does not do so in BAEC. This implies that...
regulation of eNOS expression levels by steroids takes place on an endothelial cell subtype-specific fashion. Nonetheless, BAEC preexposed to dexamethasone exert attenuated levels of VEGF-stimulated nitric oxide production compared with vehicle control cells, despite total eNOS protein expression levels being constant. Earlier studies provided several other mechanisms whereby steroids may lead to dysfunction of eNOS system. These include down-regulation of eNOS mRNA/protein (38, 39), tetrahydrobiopterin insufficiency leading to eNOS “uncoupling” (28), or down-regulation of glucose-6-phosphate dehydrogenase activity (20). Thus our results may now identify an additional point of regulation at which these steroid receptor agonists decrease nitric oxide production even at the situation where they do not alter total eNOS abundance, provided that caveolin-1 has been established as a key inhibitory binding partner of eNOS protein (27). In our system, simple transfection with caveolin-1 specific siRNA did not increase basal NO production with a statistical significance. This may be due to the degrees of caveolin-1 protein knockdown, the sensitivity of our detection method, or some other experimental conditions.

In summary, we have demonstrated that pharmacologically relevant doses of GR and MR agonists induce caveolin-1, a major regulatory protein of plasmalemmal caveolae, in cultured vascular endothelial cells. Cells pretreated with dexamethasone that express higher levels of caveolin-1 exhibit attenuated responses to a polypeptide growth factor VEGF at the levels of signal transduction, cell migration, and tube formation. We propose that steroid-induced upregulation of caveolin-1 identifies a novel point of control at which ligands for nuclear receptors modulate signal transduction pathways activated by cell surface receptors in vascular endothelium. Because treatment with dexamethasone appears to augment caveolin-1 expression in vascular endothelium in living animals as well, our study may also provide another mechanism underlying cardiovascular disorders associated with perturbation of steroid hormone systems.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


